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TetR Is a Positive Regulator of the Tetanus Toxin Gene in *Clostridium tetani* and Is Homologous to BotR

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The TetR gene immediately upstream from the tetanus toxin (TeTx) gene was characterized. It encodes a 21,562-Da protein which is related (50 to 65% identity) to the equivalent genes (*botR*) in *Clostridium botulinum*. TetR has the feature of a DNA binding protein with a basic pI (9.53). It contains a helix-turn-helix motif and shows 29% identity with other putative regulatory genes in *Clostridium*, i.e., *uviA* from *C. perfringens* and *txeR* from *C. difficile*. We report for the first time the transformation of *C. tetani* by electroporation, which permitted us to investigate the function of *tetR*. Overexpression of *tetR* in *C. tetani* induced an increase in TeTx production and in the level of the corresponding mRNA. This indicates that TetR is a transcriptional activator of the TeTx gene. Overexpression of *botR*/*A* (60% identity with TetR at the amino acid level) in *C. tetani* induced an increase in TeTx production comparable to that for overexpression of *tetR*. However, *botR*/*C* (50% identity with TetR at the amino acid level) was less efficient. This supports that TetR positively regulates the TeTx gene in *C. tetani* and that a conserved mechanism of regulation of the neurotoxin genes is involved in *C. tetani* and *C. botulinum*.

Tetanus toxin (TeTx) and botulinum neurotoxins (BoNTs) are the most potent protein toxins. They have similar structures and modes of action at the molecular level, and they are synthesized as single-chain proteins (approximately 150 kDa) which are proteolytically activated to dichain derivatives involving a light chain (L) (approximately 50 kDa) and a heavy chain (H) (approximately 100 kDa). Both chains remain linked by a single disulfide bridge. In the culture supernatants and contaminated food, BoNTs are associated with nontoxic proteins (ANTPs) to form complexes whose molecular sizes range from 230 to 900 kDa. In contrast, TeTx does not form such complexes (21). Certain ANTPs are hemagglutinins (HA). The TeTx and BoNT genes in various strains have been characterized. In *Clostridium botulinum*, the BoNT genes are localized in the 3′ part of the *C. botulinum* locus and are preceded by the gene encoding the nontoxic, non-HA component (NTNH). The HA genes lie upstream of the NTNH-BoNT genes and are transcribed in the opposite orientation. A gene encoding a 21- to 22-kDa protein is localized in the 5′ part of the *C. botulinum* locus in *C. botulinum* C and D (11, 17) or between the NTNH-BoNT and HA genes in *C. botulinum* A, B, F, and G (1, 5, 6, 12, 13). This protein called BotR shows the feature of a transcriptional regulator (basic pI [10.4] and the presence of helix-turn-helix motifs), and it is related (25 to 28% identity according to the Bestfit program) to other regulatory proteins such as UviA from *Clostridium perfringens* (10), a protein (TxeR) from *Clostridium difficile* (16), and, to a lesser extent, MsmR from *Streptococcus mutans* (20). The *txeR* gene is located directly upstream from the *tcdB* and *tcdA* genes encoding the *C. difficile* toxins B and A, respectively, which are responsible for the gastrointestinal disorders caused by this bacterium. It was shown to be a positive regulator of *tcdB* and *tcdA* gene promoters in *Escherichia coli* (16).

In *Clostridium tetani*, a gene homologous to *botR* was found in the flanking regions of the TeTx gene. Thus, it was reported that a DNA sequence upstream of the TeTx gene encodes 29 C-terminal amino acids homologous to BotR (1, 7, 8). No gene related to those encoding NTNH and HA components of botulinum complexes was detected. Here, we report the complete characterization of *tetR* from *C. tetani*, and we report that TetR and also BotR from *C. botulinum* A (BotR/A) and *C. botulinum* C (BotR/C) are positive regulators of TeTx gene expression in *C. tetani*.

MATERIALS AND METHODS

Bacterial strains and plasmids. *C. tetani* CN655 and recombinant strains were grown in broth containing trypticase (30 g/liter), yeast extract (20 g/liter), glucose (5 g/liter), and cysteine-HCl (0.5 g/liter) (pH 7.2) under anaerobic conditions. *Clostridium* DNA was extracted and purified as previously described (18).

DNA techniques. Ligation, transformation, sequencing, and preparation of plasmid DNA from *E. coli* were conducted by standard procedures (22).

Transformation of *C. tetani* by electroporation. Competent cells from *C. tetani* CN655 were prepared in an anaerobic chamber. The bacteria of a Trypticase-glucose-yeast extract (TGY) culture (100 ml) were recovered by centrifugation in the middle of the exponential growth phase, washed in distilled water, and suspended in 0.5 ml of 7 mM Na_2HPO_4 (pH 7.4) containing 1 mM $MgCl_2$ and 270 mM sucrose. Plasmid DNA (1 to 5 μg) produced in *E. coli* HB101 was added to 50 μl of cell suspension. Electroporation was performed outside the anaerobic chamber with a Bio-Rad gene pulser (2.5 kV, 200 Ω, and 25 μF) and a hermetically sealed cuvette with an anaerobic atmosphere. The bacteria were diluted in TGY, incubated for 3 h at 37°C, and plated onto TGY agar containing 5 μg of erythromycin per ml in an anaerobic chamber.

Construction of plasmids for expression of *tetR* and *botR*/*C* gene expression plasmids. A DNA fragment containing the coding region of *tetR* was amplified by PCR from *C. tetani* CN655 with primers introducing a *Nco*I site at the translational start codon and a *Pst*I site immediately downstream of the stop codon. The amplification product cut by *Nco*I and *Pst*I was cloned into the high-copy-number vector pAT19 downstream of the *C. perfringens* iota toxin gene promoter and upstream of the 3′ part of the iota toxin *ibp* gene as previously described (pMRP306) (15). The resulting plasmid, pMRP365, was transferred into *C. tetani* CN655 yielding the CN655-OE strain.

A similar construction was done with *botR*/*C*. The coding region of *botR*/*C* was amplified by PCR from *C. botulinum* C 468 (11) by adding *Nco*I and *Pst*I sites at

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the 5′ and 3′ parts, respectively, and was cloned into pMRP306 digested with *Nco*I-*Pst*I. The resulting plasmid (pMRP319) was transferred into *C. tetani* CN655, yielding CN655-BotR/C.

RNA isolation and RNA dot blots. Total RNA was extracted from *C. tetani* cultures in the middle of the exponential growth phase by using Trizol (Gibco-BRL, Cergy Pontoise, France). The bacterial pellet from a 10-ml culture (optical density at 600 nm [OD_{600}], 1.6 to 1.8) was washed twice in distilled water and suspended in 200 μl of 10 mM Tris-HCl (pH 7)–10 mM EDTA–20% sucrose containing 1 mg of lysozyme. The mixture was incubated for 30 min at 37°C and centrifuged. The pellet was suspended in 1 ml of Trizol, and the suspension was incubated for 5 min at room temperature. The subsequent steps were performed according to the manufacturer's recommendations.

Serial dilutions of total RNA in 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) were transferred onto Hybond N^+ membranes (Amersham, Paris, France). The membranes were incubated at 60°C for 2 h in Rapid Hybridization Buffer (Amersham), with the PCR-amplified fragments corresponding to the TeTx gene which were ^{32}P labeled with the Megaprime kit (Amersham). The membranes were washed in 0.1× SSC–0.1% sodium dodecyl sulfate at 60°C and exposed to X-ray films.

PAGE and immunoblotting procedure. Proteins were precipitated from the supernatant of cultures (OD, 1.8) of wild-type and recombinant *C. tetani* strains with 10% trichloroacetic acid. The precipitate was collected by centrifugation and was washed with acetone. The solubilized proteins in 50 mM Tris-HCl (pH 8) and serial dilutions were subjected to SDS-polyacrylamide gel electrophoresis (PAGE) in a 10% acrylamide gel.

The immunoblotting procedure of Burnette (2) was used. Proteins separated by 0.1% SDS–10% PAGE were transferred electrophoretically to nitrocellulose sheets (Hybond C; Amersham). The nitrocellulose sheets were incubated for 1 h in phosphate-buffered saline containing 5% dried milk and were then incubated overnight at room temperature with a 1:400 dilution of rabbit anti-TeTx antibodies. Bound antibodies were detected with peroxidase-labeled protein A and the chemiluminescence kit provided by Amersham.

Toxicity to mice. Serial twofold dilutions of samples (0.5 ml) in 50 mM sodium phosphate buffer (pH 6.3) containing 0.2% (wt/vol) gelatin were injected intraperitoneally into mice weighing 18 to 20 g. Four mice were used for each dilution. The mice were observed over 4 days, and the numbers that died were recorded.

Nucleotide sequence accession number. The nucleotide sequence reported in this paper has been submitted to the EMBL Data Library under accession no. AJ006534.

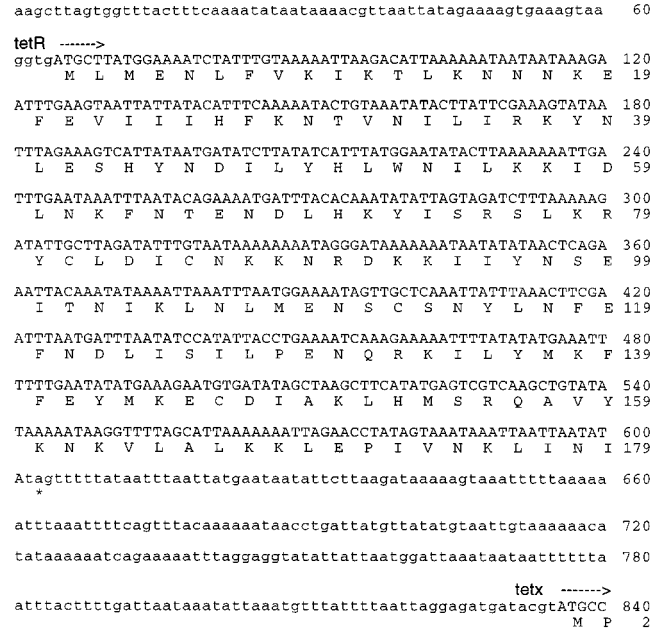

FIG. 1. Nucleotide and deduced amino acid sequences of TetR. The amino acid sequence is shown in single-letter code below the nucleotide sequence. The nontranslated regions upstream of the *tetR* and between the *tetR* and TeTx open reading frames are indicated by lowercase letters. Arrows identify the translational start codons of *tetR* and the TeTx gene.

RESULTS

Characterization of the *tetR* gene. Previous reports of the TeTx nucleotide sequence indicated the presence of *tetR* immediately upstream of the TeTx gene (7, 8). To obtain the complete sequence of *tetR*, we cloned a 2.4-kbp *Hin*dIII/*Eco*RI fragment and established the sequence of an area of 480 bp located upstream of the previously reported sequence (8). The nucleotide sequence and the deduced amino acid sequence of *tetR* are presented in Fig. 1. TetR has a calculated molecular mass of 21,562 Da and consists of 178 amino acids. It displays 50 to 65% identity with the corresponding proteins of *C. botulinum* strains. A sequence alignment is shown in Fig. 2. TetR exhibits the characteristic features of a DNA binding protein, i.e., a calculated basic pI (9.53) and a helix-turn-helix motif, and shows significant homology to UviA from *C. perfringens* and TxeR from *C. difficile*, two other putative *Clostridium* regulatory proteins (Fig. 2).

Overexpression of the *tetR* gene in *C. tetani*. To analyze the function of TetR, the *tetR* gene was overexpressed in *C. tetani*. A truncated promoter region of *tetR* was present only in the 2.4-kbp *Hin*dIII-*Eco*RI fragment and was too short to permit expression of the *tetR* gene. Thus, the coding region of *tetR* was amplified by PCR and cloned into the vector containing the promoter of the *C. perfringens iap* gene, which was used for *Clostridium* gene expression (15). The resulting plasmid, pMRP365, was transferred by electroporation into *C. tetani* CN655, yielding CN655-OE.

TeTx production in the wild-type (CN655) and recombinant (CN655-OE) strains was monitored by measuring mouse lethal activity. As shown in Fig. 3, the lethal activity in the culture supernatant during the exponential growth phase was higher (six to eight times) in CN655-OE than that in the wild-type strain. The increase in TeTx production by CN655-OE was confirmed by Western blotting with specific antibodies against TeTx (Fig. 4). Transfection of pAT19 did not result in an increase in TeTx production compared with the wild type (data not shown). These data indicate that the *tetR* gene in high copy number and in *trans* position in *C. tetani* induced an increase in TeTx production. No other extracellular protein seemed to be overproduced, as shown in Fig. 5.

The specific mRNA of the TeTx gene was assayed by dot blot analysis in the wild-type and recombinant CN655-OE strains. The amounts of TeTx gene-specific mRNAs were approximately four times higher than those in the wild-type strain (Fig. 6). This was in agreement with the increase in the level of TeTx in culture supernatant quantified by mouse lethal activity and immunoblotting. These results show that TetR activates TeTx expression at the transcriptional level.

Overexpression of *botR*/*A* and *botR*/*C* genes in *C. tetani*. Since TetR shows a high similarity to the equivalent regulatory genes (*botR*s) from *C. botulinum*, it was interesting to know if these genes of the same family can be functionally interchangeable. BotR/A shows an overall identity of 60% with TetR (Fig. 2). It was found that plasmid pMRP309 containing the *botR*/*A* gene under the control of its own promoter induced a significant increase in the production of BoNT/A and ANTPs and in the corresponding mRNAs in *C. botulinum* A (15). *C. tetani* CN655 was transformed by electroporation with pMRP309, yielding strain CN655-BotR/A. A comparable increase in the amount of TeTx assayed by the mouse test and immunoblotting was observed during the exponential growth phase of CN655-BotR/A and CN655-OE (Fig. 3 and 4). Mouse lethal activity in the culture supernatant of CN655-OE and CN655-botR/A was approximately eight times higher than that in the wild-type strain (Fig. 3). The overproduction of TeTx assayed by immunoblotting was increased by approximately 16 times in CN655-OE and 8 times in CN655-botR/A compared to the wild-type strain (Fig. 4). Moreover, the amounts of mRNAs of the TeTx

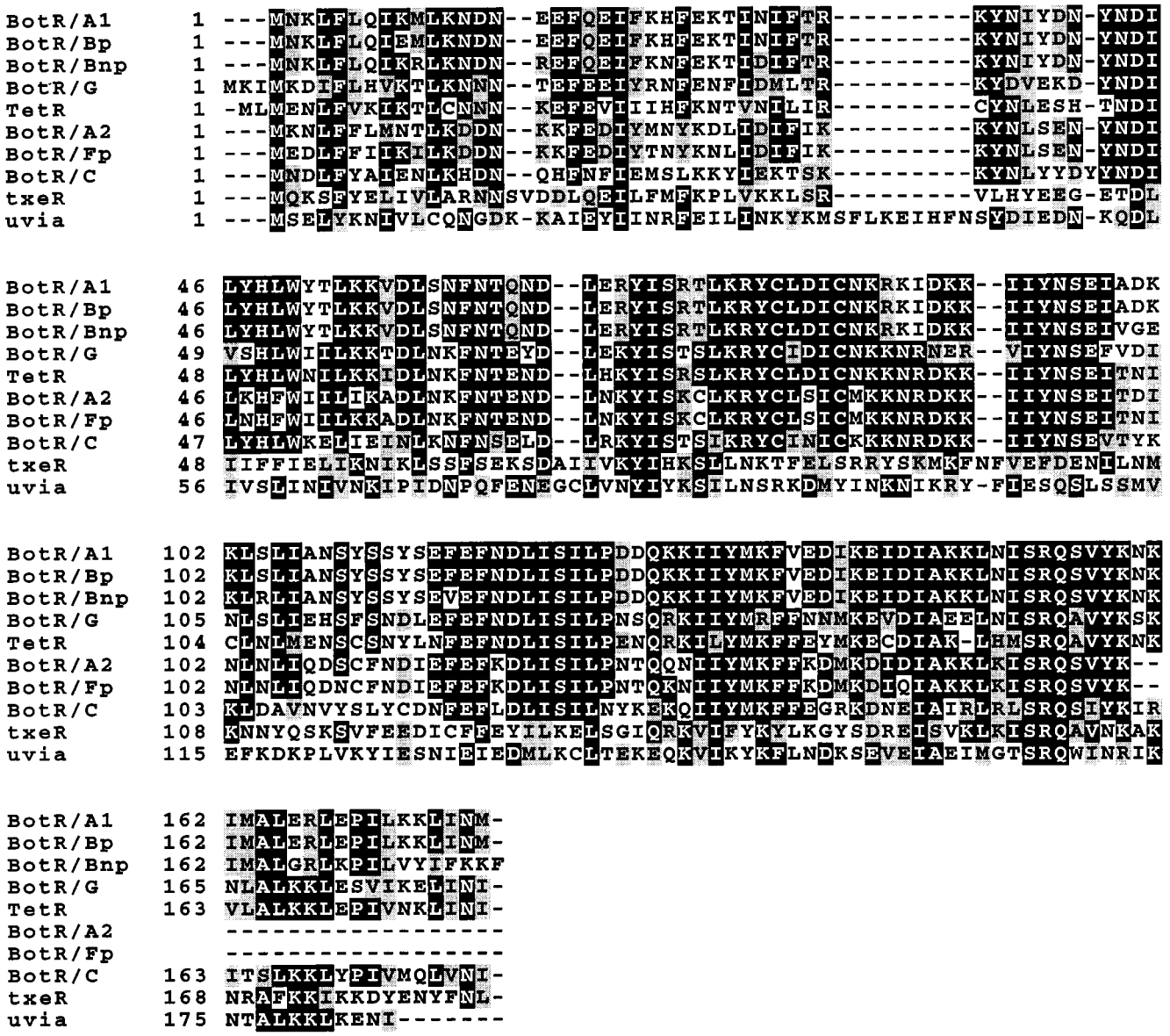

FIG. 2. Alignment of TetR; BotRs from *C. botulinum* A1, A2, B proteolytic (Bp), B nonproteolytic (Bnp), C, F proteolytic (Fp), and G; and the related regulatory proteins from *C. difficile* (TxeR) and *C. perfringens* (UviA).

gene were increased (approximately four times) in both strains (Fig. 6). This indicates that BotR/A was able to positively regulate the TeTx gene in *C. tetani*.

The potential effect of BotR/C, which is less related to TetR at the amino acid level (50% identity) than BotR/A (60% identity), in *C. tetani* was investigated. Plasmid pMRP319, corresponding to the pAT19 vector containing the coding region of *botR/C* under the control of the *iap* gene promoter (15), was transferred into *C. tetani* CN655 by electroporation (CN655-BotR/C). The production of TeTx assayed by mouse lethal activity was three times higher in CN655-BotR/C than that in the wild-type strain and was eight times higher as determined by immunoblotting (Fig. 3 and 4). No significant increase in TeTx-specific mRNA was detected in CN655-BotR/C (Fig. 6). This shows that *botR/C* stimulated the expression of the TeTx gene, albeit at a lower extent than *botR/A*.

DISCUSSION

We report the complete sequence of the *tetR* gene from *C. tetani*, which is highly related to the *botR* genes from *C. botulinum* A, B, C, D, F, and G (1, 5, 6, 12, 13). TetR shows an overall level of identity of from 50% with BotR/C to 65% with BotR/F. This family of genes is related to other putative regulatory genes in *Clostridium*, such as *uviA* in *C. perfringens* and *txeR* in *C. difficile* (10, 16). TetR and the other related proteins possess the features of DNA binding proteins, i.e., high pI (pH 9.53) and the presence of a helix-turn-helix motif.

We succeeded in transforming *C. tetani* with pAT19, which is a shuttle vector between gram-positive and gram-negative bacteria and which contains a replication origin from *Enterococcus fecalis* (23). The electroporation conditions were similar to those used for the *C. botulinum* transformation (15). The present article is the first report of genetic transformation in *C. te-*

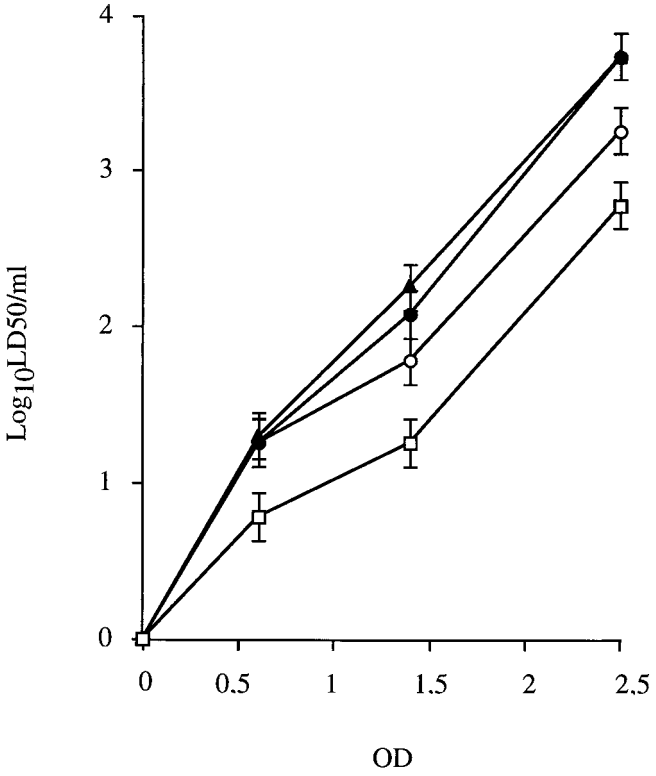

FIG. 3. Mouse lethal activity in culture supernatants of wild-type *C. tetani* CN655 (□) and recombinant strains overexpressing the *tetR* (▲), *botR/A* (●), and *botR/C* (○) genes. The mouse lethal activity (LD_{50}) is plotted against the OD_{600} for each culture. The means and standard deviations of the values from two experiments are indicated.

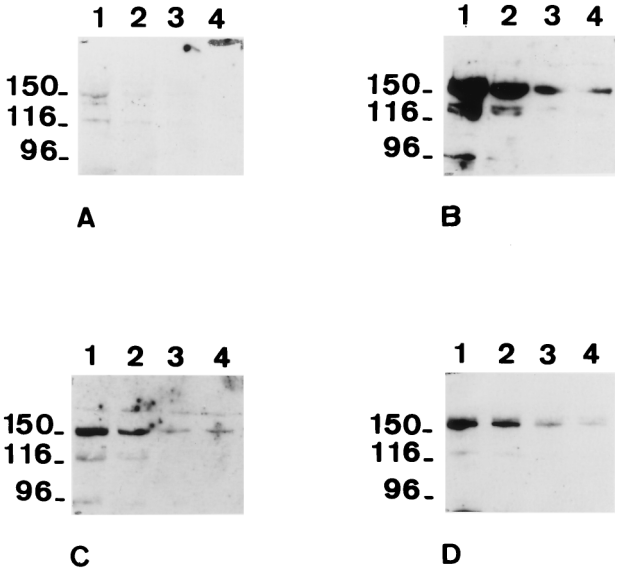

FIG. 4. Production of TeTx assayed by Western blotting with anti-TeTx antibodies in wild-type *C. tetani* CN655 (A) and in recombinant strains overexpressing the *tetR* (CN655-OE) (B), *botR/A* (CN655-BotR/A) (C), and *botR/C* (CN655-BotR/C) (D) genes. Supernatants of each culture (OD_{600}) were concentrated by trichloroacetic acid precipitation, 20 μg of protein was loaded on lane 1, and serial twofold dilutions were loaded in the subsequent lanes. In panels B and D, the upper bands correspond to the whole TeTx and the lower bands correspond to the H chain.

tani and construction of recombinant *C. tetani* strains to investigate a gene function in this microorganism.

Overexpression of *tetR* in *trans* position induces an increase in TeTx production, as monitored by mouse lethality in culture supernatant and by Western blotting. A corresponding increase in the specific mRNA of the TeTx gene indicates that *tetR* positively regulates the transcriptional level of the toxin gene in *C. tetani*. It can not be ruled out that *tetR* in *cis* position could be more efficient. *tetR* seems to regulate specifically the TeTx gene and to have no pleomorphic effect. We explored if the equivalent genes (*botR*) from *C. botulinum* are functional in *C. tetani*. The high-copy-number vector pAT19 containing *botR/A* or *botR/C* was transferred by electroporation into *C. tetani* CN655. BotR/A, which is more closely related to TetR than BotR/C, produced a higher increase in TeTx production and in the specific mRNA, compared with BotR/C. This shows that BotR/A and BotR/C are functional in *C. tetani*. The different levels of effect between BotR/A and BotR/C could be due to a lower level of expression of *botR/C*, since *botR/C* was under the control of the *iap* promoter (pMRP365) and *botR/A* was under the control of its own promoter (pMRP309). However, *tetR* was constructed under the control of the *iap* promoter and induced an equivalent activation of TeTx gene expression equivalent to that of *botR/A*. The more distant relatedness of BotR/C to TetR than to BotR/A could explain the reduced efficiency of BotR/C in *C. tetani*. These data suggest a common mechanism of regulation of the neurotoxin genes in *C. tetani* and *C. botulinum*.

We found that BotR/A stimulates expression of both the BoNT and ANTP genes (15). The −10 and −35 regions of the neurotoxin and ANTP gene promoters in *C. botulinum* A, B, C, D, F, and G and *C. tetani* contain conserved sequences (1, 12). Moreover, BotR/A seems to interact directly with the promoter region and the conserved motifs could represent binding sites for the regulatory proteins (15). TetR could be also a regulatory protein which binds the promoter region of the TeTx gene. Whether Tet/R and BotR are involved in a cascade of regulatory proteins is unknown. It has been found that short peptides from casein hydrolysates are important for toxinogen-

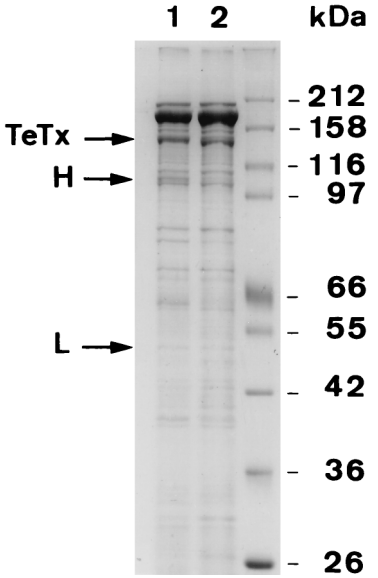

FIG. 5. PAGE of extracellular proteins (50 μg) of recombinant strain overexpressing *tetR* (CN655-OE) (lane 1) and of *C. tetani* wild-type CN655 (lane 2). H and L, heavy and light chains of TeTx, respectively.

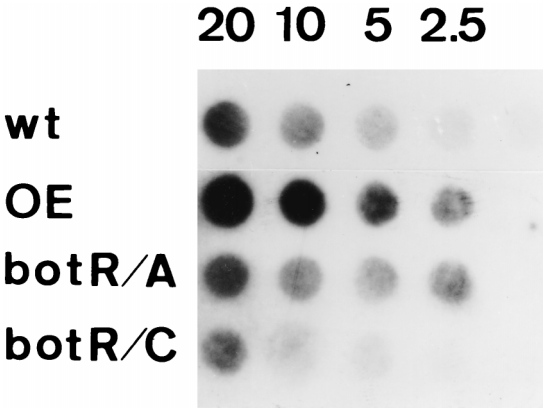

FIG. 6. mRNA dot blots from the wild-type strain CN655 (wt) and from recombinant strains CN655-OE (OE), CN655-BotR/A (botR/A), and CN655-botR/C (botR/C) with probes specific for the TeTx gene. The mRNA was prepared from cultures at an OD_{600} of 1.4. The total amounts of mRNA loaded in each lane are indicated in micrograms.

esis in *C. tetani* (19), but the environmental signals which trigger neurotoxin production remain to be determined.

The presence of highly conserved genes in the close vicinity of the clostridial neurotoxin genes, which are functionally interchangeable, constitutes additional evidence that the locus of clostridial neurotoxin genes derived from a common ancestor. However, the NTNH and HA genes, which lie upstream from the BoNT genes in the different *C. botulinum* toxinotypes, are missing in *C. tetani*. The *tetR* gene is the only ANTP gene which was found in *C. tetani*.

Vaccination against tetanus is extremely effective in preventing this disease, and widespread vaccination has almost eradicated tetanus from developed countries. Current tetanus vaccines are produced by formaldehyde treatment of TeTx produced by wild-type *C. tetani* to yield the immunogenic toxoid. A novel generation of tetanus vaccines involves production of the C-terminal part (fragment C) of TeTx, which is nontoxic and is able to induce neutralizing antibodies. The production of large quantities of recombinant fragment C in various organisms such as *E. coli*, *Lactococcus lactis*, *Baculovirus*, and *Pichia pastoris* (3, 4, 9, 14, 24) was attempted. Our findings on the genetic transformation of *C. tetani* and on the identification of TetR as a positive regulator open the possibility of using *C. tetani* as an engineering system for vaccine production. It may be possible to construct *C. tetani* strains which produce large amounts of TeTx or fragment C. *C. tetani* has the advantage of secreting a soluble form of TeTx, and this organism is already used in industrial fermentation.

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